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14. ABSTRACT

We developed an in vitro injury model of stroma cultured from bone marrow samples of mice and human donors. We demonstrated that oxidative and hypoxic damage and estrogen deprivation can induce IL-6 and IL-8 export by human female, premenopausal stroma. We adapted a breast cancer cell co-culture model to determine effect of injury on stromal capacity to support dormancy and demonstrated that breast cancer cells induce inflammatory cytokine secretion by stroma. We adapted an osteoclast activation model to determine the capacity of injured stroma to activate osteoclasts. We also adapted a preosteoblast co-culture model to determine the role of osteoblasts in the micrometastatic cell niche. Studies on estrogen replacement and anti-inflammatory treatment will determine reversibility.

15. SUBJECT TERMS
Bone marrow stroma, senescence, micrometastases, breast cancer, interleukin 6, osteoclast

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INTRODUCTION:

The proposed project investigates one potential mechanism for the reawakening of microscopic breast cancer metastases in the bone marrow of patients at significant periods of time after initial treatment for local disease. We are testing the hypothesis that deprivation of estrogen that occurs after menopause may be a contributing factor to the generation of senile senescence in the bone marrow stroma of women. In this model, inflammatory cytokines generated by secretory senescent stromal cells induce dormant breast cancer cells to re-enter the cell cycle and grow into recurrent tumors in the bone.

We began testing this hypothesis by defining the secretory senescence phenotype in murine stroma initially using hypoxic and oxidative stress, and subsequently using estrogen deprivation. We developed *in vitro* stromal injury model in bone marrow stroma with hypoxia, oxidative stress and estrogen deprivation as defined by IL-6 and IL-8 export and activation of the TGFβ pathway. We began developing co-cultivations methods for determining the effects of stromal injury on dormancy and proliferation of estrogen sensitive breast cancer cells. We also adapted methodology to determine the effect of injured stroma on osteoclast stimulation. Finally, we began to test a hypothesis that the niche harboring dormant breast cancer cells in the bone marrow involves osteoclast precursors that were shown to serve that role in hematopoietic stem cell survival. We also began to address the specific niche in the microenvironment responsible for support of the micrometastatic dormant clone. We used preosteoblast cell line to co-culture breast cancer cells alone and in mixing experiments with stromal cells. Our preliminary experiments demonstrated that osteoblasts promote the growth of both growing as well as dormant clones. Confirmation of these studies and determining mechanisms for these effect will be key in understanding the survival effects of the niche on dormant micrometastases. The characteristic cancer repopulating capacity of breast cancer cells forming dormant clones on osteoblasts will be key in addressing this specific, chemo and radioresistant population. In vivo studies with ovariectomized mice and xenograft tumors are ongoing to determine if stromal senescence in vivo promotes growth of micrometastases in the bone marrow.

BODY:

Initial studies were conducted in murine stroma. We developed a stromal culture model from murine bone marrow obtained from female Ncr nu/nu mice. Bone marrow was cultured on 25 cm^2 flasks in Gartner's Medium until a stromal layer formed at the bottom of the flask. Stromal cells were trypsinized and distributed to 24 well plates and cultured until confluent.

We first determined the effects of the timing and dosage of hydrogen peroxide (H_2O_2), an agent that generates oxidative stress and of carbonyl-cyanide m-chlorophenylhydrazzone (CCCP) an agent that blocks the mitochondrial electron transport chain, to create hypoxic stress, on injury to stroma as determined by lactate dehydrogenase (LDH) release. Figure 1 demonstrates the effects of 10, 100 and 1,000 μM H_2O_2 and Figure 2 the effects of 10, 100 and 1,000 μM CCCP on the relative increase in LDH in the media at 24, 48, 72 hours and six days after a one hour incubation. Cytotoxicity was achieved by 24 hours with both 100 μM H_2O_2 and CCCP by 24 hours but the effects of the injury began to ebb by 3 to 7 days, probably because the injured cells had died already. These experiments were done to design positive control conditions for stromal injury.

Next, we developed an ELISA assay to measure export of murine IL-6 and murine IL-8 (KC) as representative assays for secretory senescence in stroma as a consequence of treatment with H_2O_2 and CCCP. We determined the effects of the timing and dosage of H_2O_2 and CCCP on the presence of IL-6 and IL-8 (KC). Figure 3 demonstrates effects of 10, 100 and 1,000 μM H_2O_2 and Figure 4, effects of 10, 100 and 1,000 μM CCCP on the concentration of IL-6 in the media at 24, 48, 72 hours and six days after a one hour incubation. Similarly, Figures 5 and 6 reflect the effects of the same treatments on the concentration of IL-8 (KC) in the same experiments.

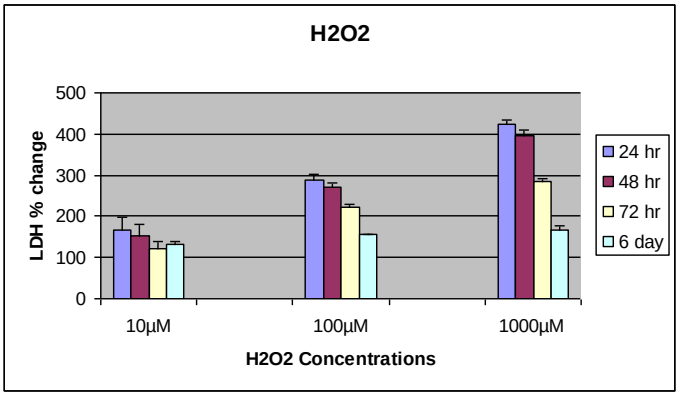


Figure 1. Time and dose effects of H_2O_2 on LDH release by murine stromal cells. Near confluent stromal monolayers cultured in 24-well plates were treated with H_2O_2 for 1 hour and then washed once with PBS. Cells were then incubated in conditioned medium with only 5% FBS. Lactate dehydrogenase (LDH) release was measured at 24h, 48h, 72h, and 6 days using the Cytotox 96 Non-Radioactive Cytotoxicity Assay (Promega) following the manufacturer's instructions. Data represent **%** LDH change of the test group compared to that of the control group. *Columns:* means; *bars:* SE. Experiments were done in triplicate at four samples per point. Data are from one representative experiment.

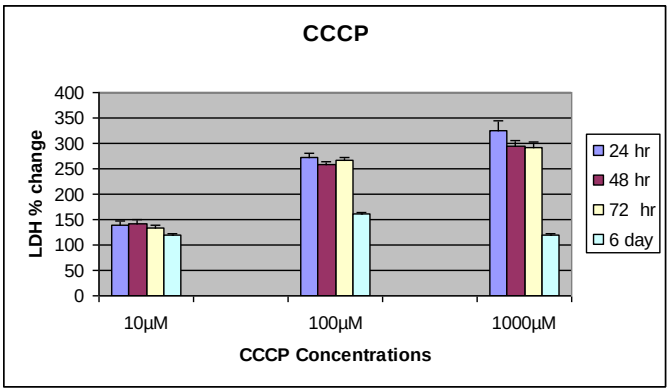


Figure 2. Time and dose effects of CCCP on LDH release by murine stromal cells. Near confluent stromal monolayers cultured in 24-well plates were treated with CCCP for 1 hour and then washed once with PBS. Cells were then incubated in conditioned medium with only 5% FBS. Lactate dehydrogenase (LDH) release was measured at 24h, 48h, 72h, and 6 days using the Cytotox 96 Non-Radioactive Cytotoxicity Assay (Promega) following the manufacturer's instructions. Data represent **%** LDH change of the test group compared to that of the control group. *Columns:* means; *bars:* SE. Experiments were done in triplicate at four samples per point. Data are from one representative experiment.

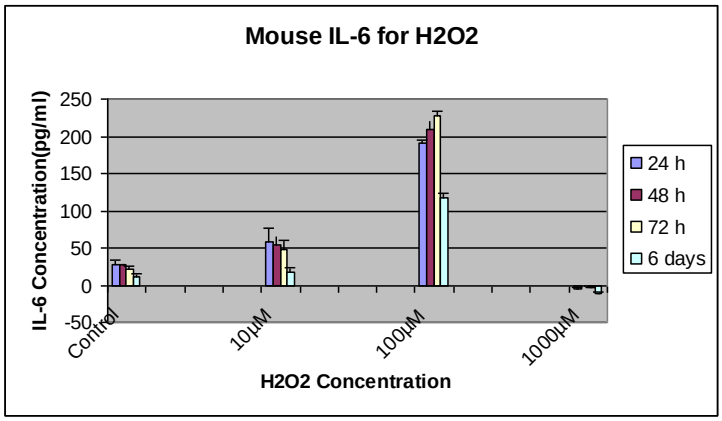


Figure 3. Time and dose effects of H_2O_2 on IL-6 in the media of murine stromal cells. Near confluent stromal monolayers cultured in 24-well plates were treated with H_2O_2 for 1 hour and then washed once with PBS. Cells were then incubated in conditioned medium with only 5% FBS. Supernatant samples were collected at 24h, 48h, 72 hours and 6 days. The IL-6 levels in the supernatant were determined using an IL-6 ELISA kit (BD Biosciences), according to the manufacturer's instructions. *Columns:* means; *bars:* SE. Experiments were done in triplicate at three samples per point. Data are from one representative experiment.

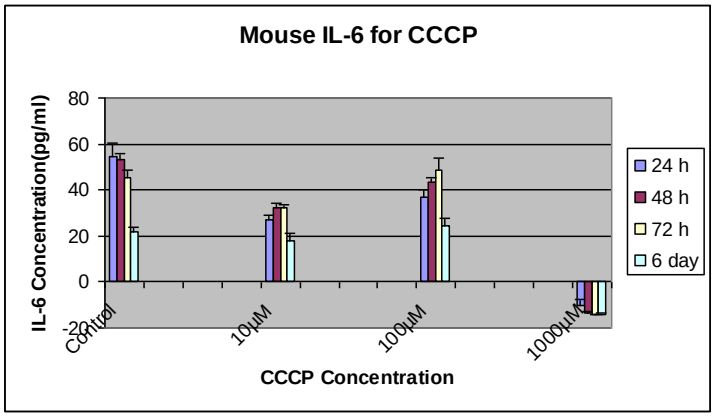


Figure 4. Time and dose effects of CCCP on IL-6 in the media of murine stromal cells. Near confluent stromal monolayers cultured in 24-well plates were treated with CCCP for 1 hour and then washed once with PBS. Cells were then incubated in conditioned medium with only 5% FBS. Supernatant samples were collected at 24h, 48h, 72 hours and 6 days. The IL-6 levels in the supernatant were determined using an IL-6 ELISA kit (BD Biosciences), according to the manufacturer's instructions. *Columns:* means; *bars:* SE. Experiments were done in triplicate at three samples per point. Data are from one representative experiment.

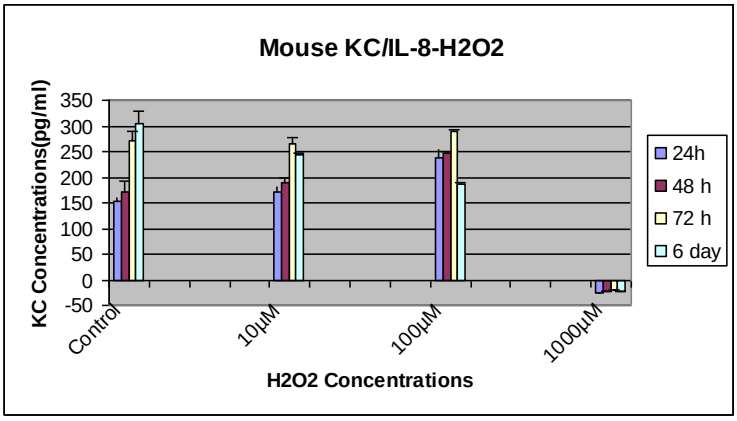


Figure 5. Time and dose effects of H_2O_2 on IL-8 in the media of murine stromal cells. Near confluent stromal monolayers cultured in 24-well plates were treated with H_2O_2 for 1 hour and then washed once with PBS. Cells were then incubated in conditioned medium with only 5% FBS. Supernatant samples were collected at 24h, 48h, 72 hours and 6 days. The IL-8 levels in the supernatant were determined using a murine IL-8 (KC) ELISA kit (R&D Systems), according to the manufacturer's instructions. *Columns:* means; *bars:* SE. Experiments were done in triplicate at three samples per point. Data are from one representative experiment.

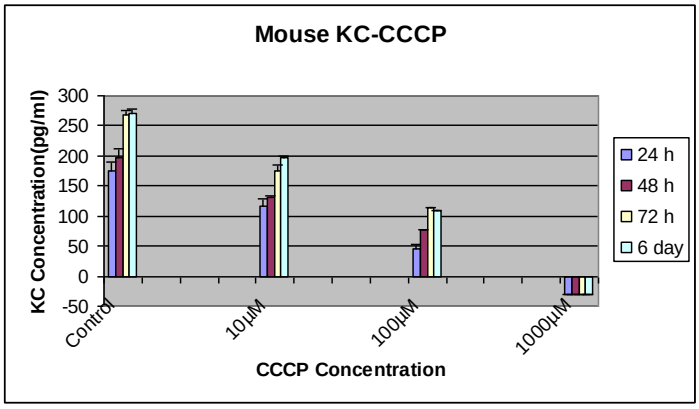


Figure 6. Time and dose effects of CCCP on IL-8 in the media of murine stromal cells. Near confluent stromal monolayers cultured in 24-well plates were treated with CCCP for 1 hour and then washed once with PBS. Cells were then incubated in conditioned medium with only 5% FBS. Supernatant samples were collected at 24h, 48h, 72 hours and 6 days. The IL-8 levels in the supernatant were determined using a murine IL-8 (KC) ELISA kit (R&D Systems), according to the manufacturer's instructions. *Columns:* means; *bars:* SE. Experiments were done in triplicate at three samples per point. Data are from one representative experiment.

IL-6 was significantly higher in stromal conditioned medium ($p<0.05$, Student's t test) 24, 48 and 72 hours after treatment with H_2O_2 10 and 100 μM. The effect ebbed after 6 days but remained significantly higher with the 100 μM treatment. Treatment with 1,000 μM H_2O_2 did not result in IL-6 secretion, likely due to outright rapid toxin-induced cell death. CCCP treatment did not result in an increase in IL-6 export to the conditioned media under any conditions assayed in mouse stroma.

Conditions for induction of IL-8 in mouse stroma differed slightly from those for IL-6 induction. Prolonged incubation of near confluent stroma from 3 to 7 days spontaneously induced significant increases in IL-8 export. After H_2O_2 transient treatment, IL-8 export to the media increased significantly from that of untreated stroma only after treatment with 100 μM H_2O_2 and only during the first two days compared with controls. Treatment with 1,000 μM H_2O_2 resulted in an immediate decrease in IL-8 in the conditioned media, sustained for 7 days, likely due to the immediate death of the cells. As with H_2O_2, CCCP treatment also did not result in an increase in IL-8 in the conditioned media over control levels.

Next, we carried out experiments with human stroma from a total of 15 normal volunteers, 13 premenopausal females and 2 male controls, under a UMDNJ and US Army IRB-approved protocol using previously published methods (Korah, et al.). Our goal of assessing stromal senescence in postmenopausal females could not be met because we did not have any postmenopausal volunteers. The data with premenopausal stroma demonstrated statistically significant differences in IL-6 export between control and CCCP-treated cells occurred during most of the time points from 1 to 28 days and estrogen-deprived stroma after a week of incubation that was sustained for the 28 days assayed in one premenopausal donor.

Confluent monolayers of human stromal cells on 24 well plates were treated with H_2O_2, CCCP and ICI182780 in quadruplicate. Rows of 4 wells were either untreated or treated with 100 μM H_2O_2, 100μM CCCP for 1 hr at 37^o, washed with PBS and then replenishes with McCoy's complete growth medium. To the fourth row, 10^{-6} M ICI182780 in phenol red-free medium was added, in which cells were maintained throughout the duration of the experiment. Supernatants from each well were collected at specified time points and stored at -80^o. ELISAs were performed on thawed conditioned media for IL-6 and IL-8 using a Human IL-6 Elisa Kit from BD & Human CXCL8/IL-8 kit from R&D Systems respectively. Control wells are the wells in which McCoy's media was replaced by DMEM/10% FCS.

Figure 7 demonstrates that statistically significant differences in IL-6 export between control and CCCP-treated cells occurred during most of the time points from 1 to 28 days in our first donor, suggesting a more sensitive response to hypoxia by human stroma than by murine stroma. It also demonstrated statistically significant differences in IL-6 export between control and estrogen deprived stroma after a week of incubation that was sustained for the 28 days assayed. This also suggests that estrogen deprivation initiates a sustained secretory pattern in human stroma at levels that relatively much higher than those in murine stroma. These data will need to be confirmed in numerous other donor volunteers, as outlined by the statistical section of our grant application. Extensive discussions and meetings with our statistician collaborator have reconciled our initial plan and the plan for data analysis, depending on whether the donor sample data distribute in a normal distribution or whether they do not, in which case transformation of the data and non parametric analysis will need to be used. In addition, we will need to analyze human and murine stroma side by side on the same dishes to compare values and confirm the suggested inferences.

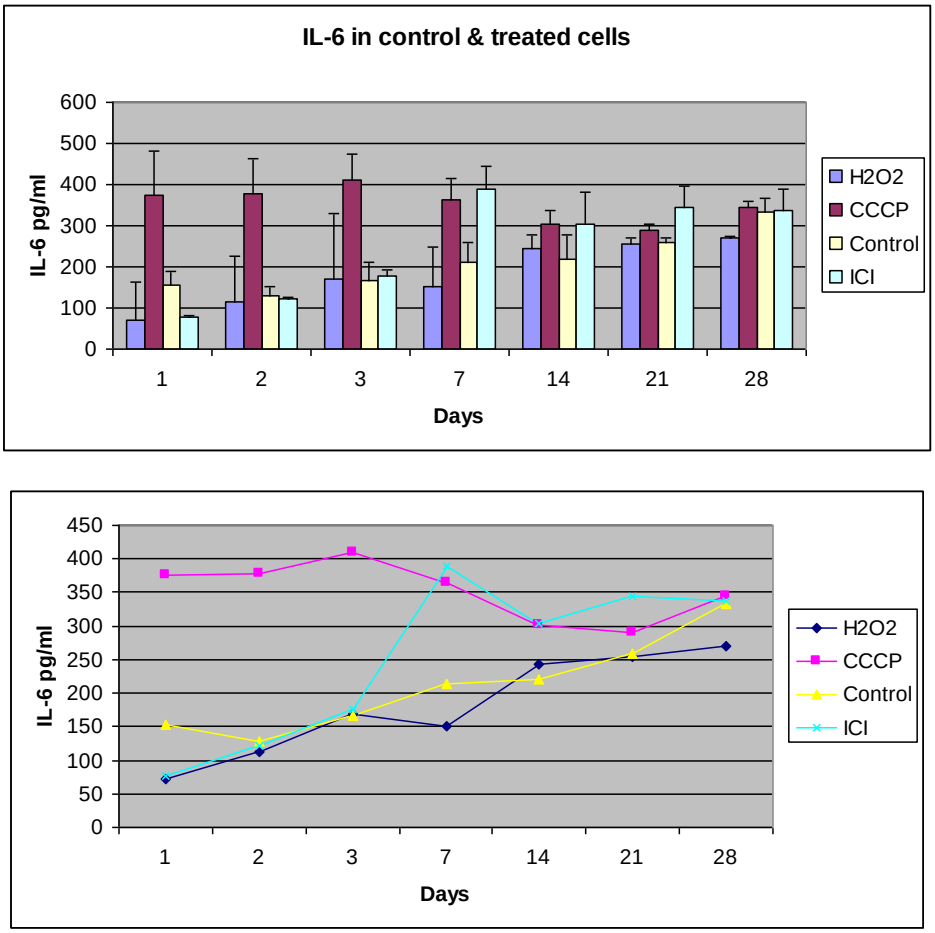


Figure 7. ELISA assay for IL-6 in a pre-menopausal human female stromal monolayers treated with oxidative, hypoxic and estrogen deprivation injury for variable time points. Quadruplicate wells of confluent monolayers of human stromal cells on 24 well plates were treated with 100 μM H_2O_2, 100 μM H_2O_2 CCCP for one hour, washed with PBS and then replenishes with McCoy's complete growth medium and incubated for variable times from 1 to 28 days, or treated and maintained in 10^{-6} M ICI182780 in phenol red-free medium throughout the duration of the experiment from 1 to 28 days. Supernatants from each well were collected at specified time points and stored at -80^{o}. ELISAs were performed of thawed conditioned media for IL-6 using a Human IL-6 Elisa Kit from BD. The bar graph represents the data and standard deviations, demonstrating statistically significant differences in IL-6 export between control and CCCP-treated cells for most of the time points and statistically significant differences between IL-6 export between control and estrogen deprived stroma after a week of incubation. The lower line graph is included to better visually demonstrate these trends. No impact was demonstrated by oxidative injury on IL-6 export.

Figure 8 demonstrates that IL-8 was not increased in human stroma as a consequence of incubations with either H_2O_2, CCCP or estrogen deprivation up until day 7. Similarly, Figure 9 demonstrates that estrogen deprivation to 28 days also did not induce IL-8 secretion by human stromal monolayers.

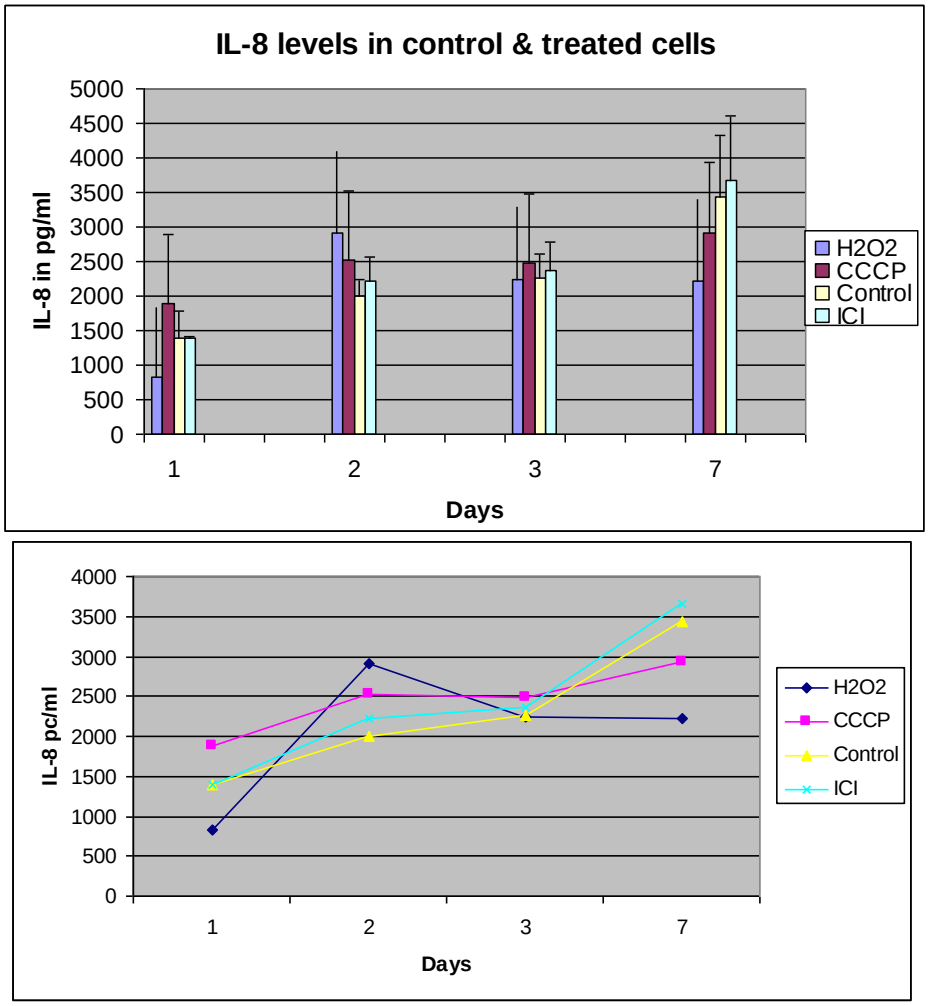


Figure 8. ELISA assay for IL-8 in a pre-menopausal human female stromal monolayers treated with oxidative, hypoxic and estrogen deprivation injury for variable time points. Quadruplicate wells of confluent monolayers of human stromal cells on 24 well plates were treated with 100 μM H_2O_2, 100 μM H_2O_2 CCCP for one hour, washed with PBS and then replenishes with McCoy's complete growth medium and incubated for variable times from 1 to 7 days, or treated and maintained in 10^{-6} M ICI182780 in phenol red-free medium throughout the duration of the experiment from 1 to 7 days. Supernatants from each well were collected at specified time points and stored at -80^{o}. ELISAs were performed of thawed conditioned media for IL-6 using a Human CXCL8/IL-8 kit from R&D Systems. The bar graph represents the data and standard deviations, demonstrating no statistically significant increases in IL-8 export between experimental and control values at all in CCCP, H_2O_2 and ICI182780 treated cells. The lower line graph is included for better visual depiction.

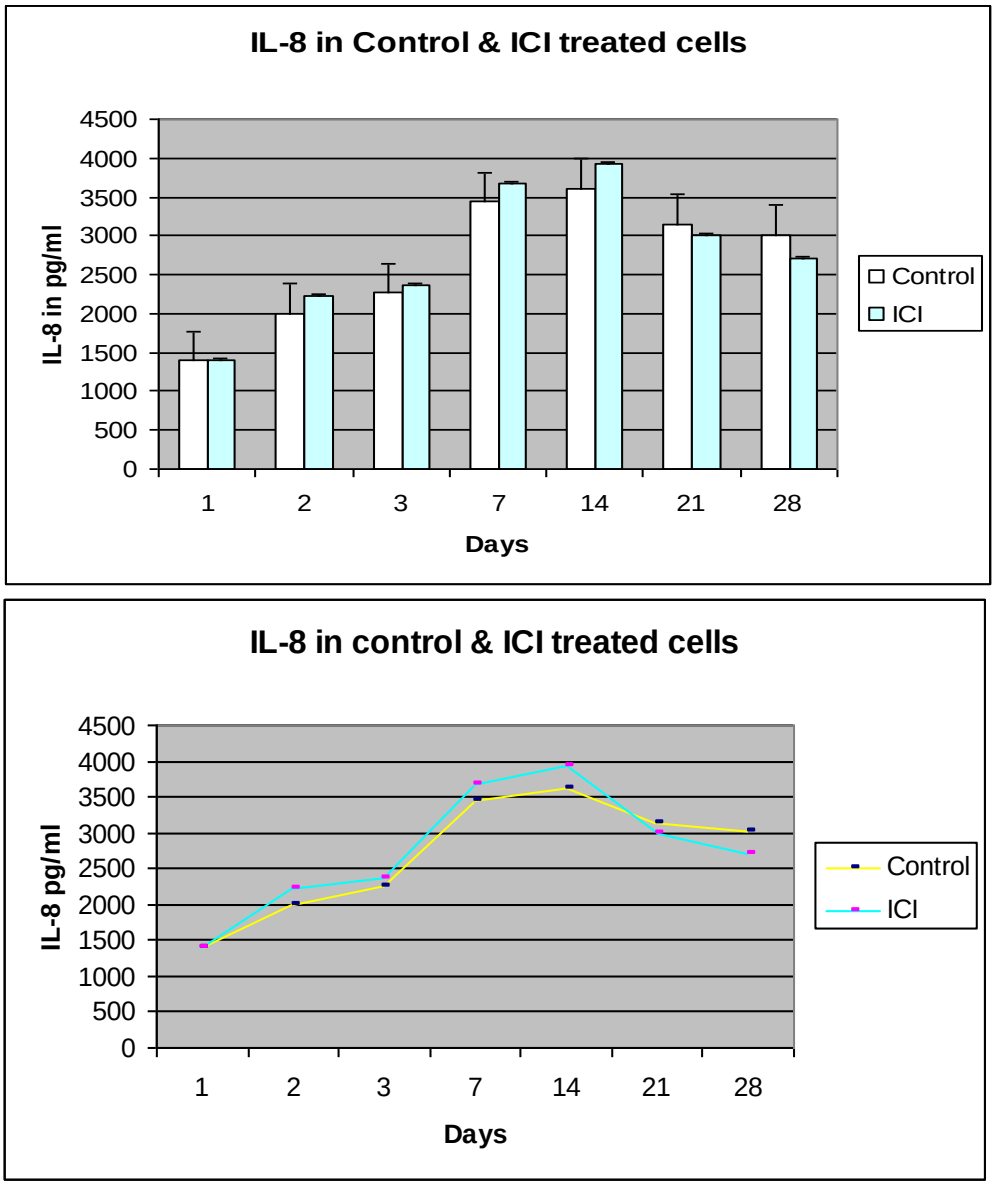


Figure 9. ELISA assay for IL-8 in a pre-menopausal human female stromal monolayers treated with estrogen deprivation injury for variable time points until 28 days. Quadruplicate wells of confluent monolayers of human stromal cells on 24 well plates were treated and maintained in 10^{-6} M ICI182780 in phenol red-free medium throughout the duration of the experiment from 1 to 28 days. Supernatants from each well were collected at specified time points and stored at -80^{o}. ELISAs were performed of thawed conditioned media for IL-6 using a Human CXCL8/IL-8 kit from R&D Systems. The bar graph represents data and standard deviations, demonstrating no statistically significant increases in IL-8 export between experimental and control values. The lower line graph is included for better visual depiction.

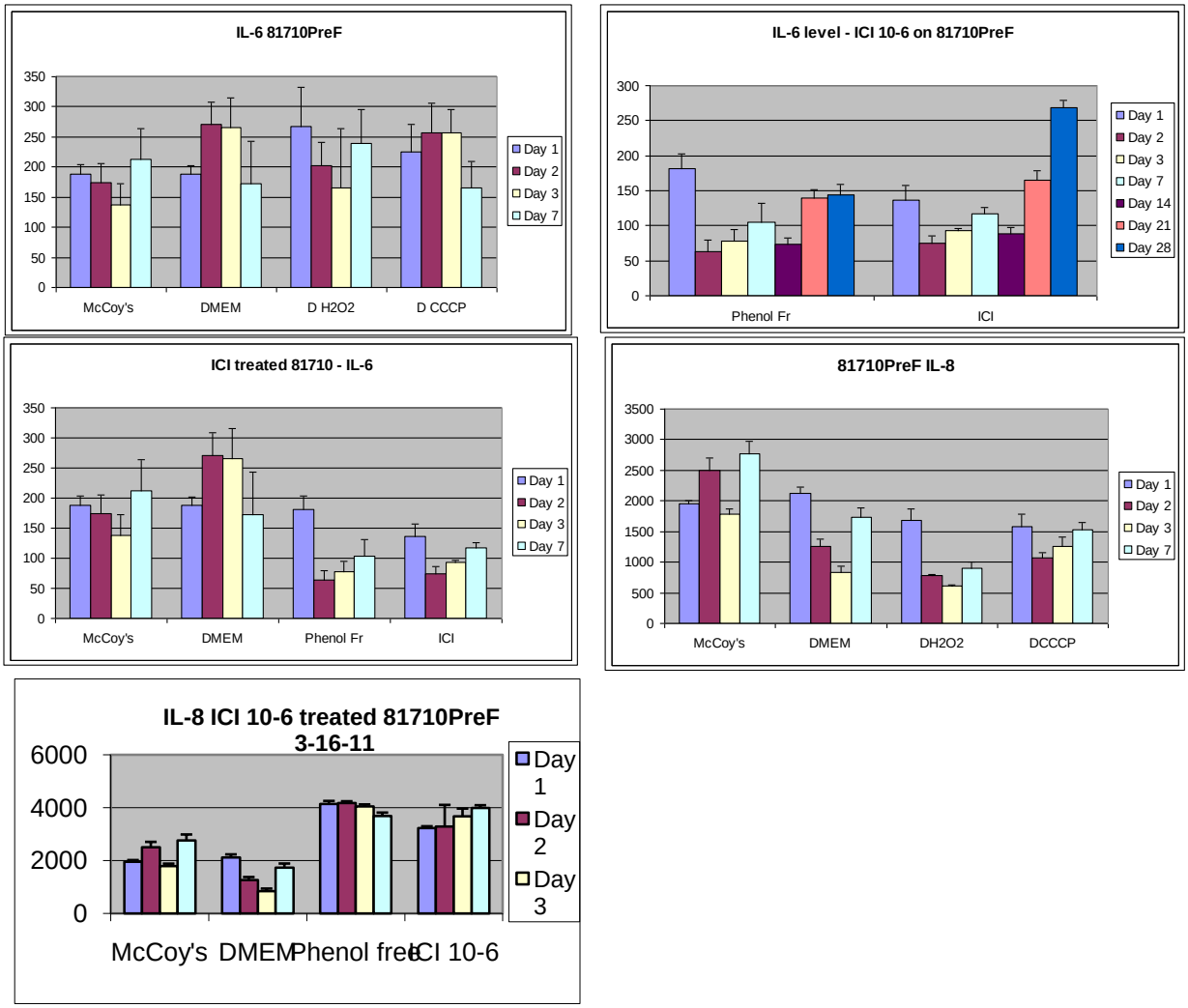
Table 1 and Figure 10 presents data from three additional female premenopausal volunteers and one male volunteer. The data appear to demonstrate a relatively consistent pattern of response, although the timing of the response varies somewhat. IL-6 in the conditioned media consistently increases with H_2O_2 and CCCP one hour treatment from 1-3 days in the three female subjects and increases with estrogen deprivation most frequently between 7-28 days. IL-8 secretion responds infrequently to oxidative and hypoxic stress but is reliably exported in response to estrogen deprivation in females. The one male assessed did not produce IL-6 nor IL-8 to any of the stimuli. It remains to be confirmed whether male and female stroma respond differently to injury under the conditions tested. Stromal samples obtained from the additional donors are in various stages of processing.

TABLE 1

	Subject	H_2O_2	CCCP	ICI182780
A.	81710PreF			
	IL-6	+ 1d	+ 1d	+ 21d, 28d
	IL-8	-	-	+ 3d, 7d
B.	121319PreF			
	IL-6	+ 1d, 2d, 3d	+ 1d, 2d, 3d	+ 7d, 28d weakly
	IL-8	-	-	+ 1, 2, 3, 7, 14, 21, 28d
C.	121710PreF			
	IL-6	+ 3d	+ 3d, 7d	+ 3, 7 and +weakly 28d
	IL-8	+ 2, 3, 3d	+ 3d weakly	+ 1, 2d
D.	111710M			
	IL-6	-	-	-
	IL-8	-	-	-

Figure 10.

A.



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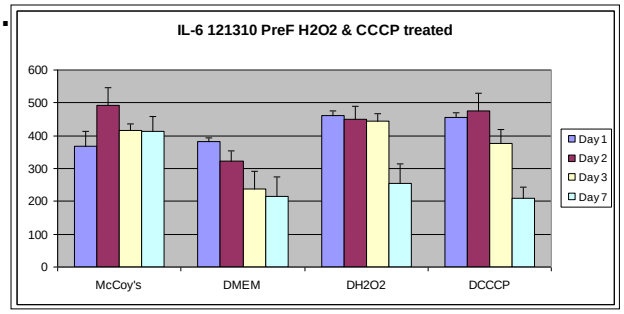
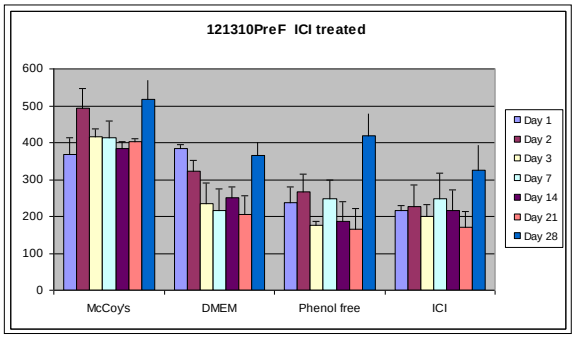
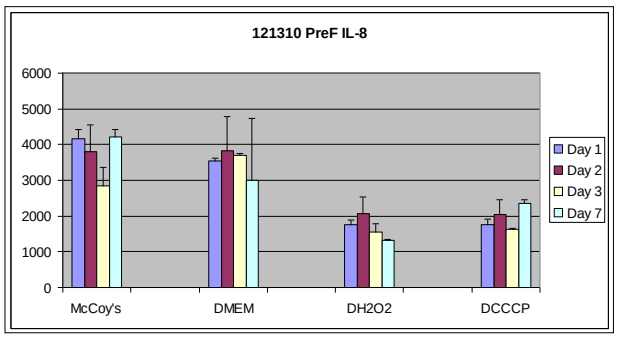
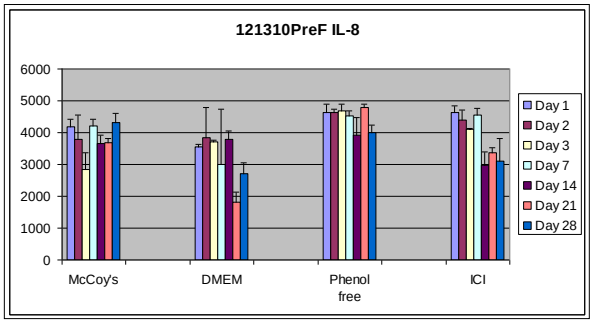
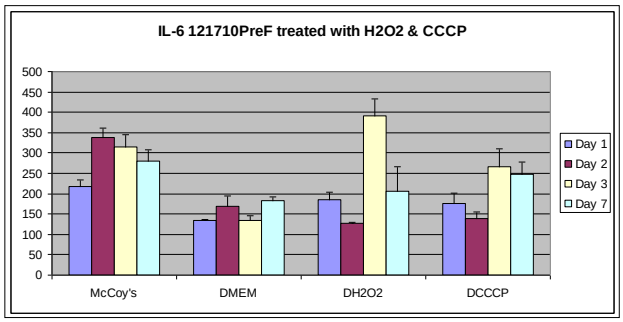

IL-6 121310 PreF H2O2 & CCCP treated
600
500
400
300
200
100
0
McCoy's
DMEM
DH2O2
DCCCP
Day 1
Day 2
Day 3
Day 7


121310PreF ICI treated
600
500
400
300
200
100
0
McCoy's
DMEM
Phenol free
ICI
Day 1
Day 2
Day 3
Day 7
Day 14
Day 21
Day 28


121310 PreF IL-8
6000
5000
4000
3000
2000
1000
0
McCoy's
DMEM
DH2O2
DCCCP
Day 1
Day 2
Day 3
Day 7


121310PreF IL-8
6000
5000
4000
3000
2000
1000
0
McCoy's
DMEM
Phenol free
ICI
Day 1
Day 2
Day 3
Day 7
Day 14
Day 21
Day 28

C.


IL-6 121710PreF treated with H2O2 & CCCP
500
450
400
350
300
250
200
150
100
50
0
McCoy's
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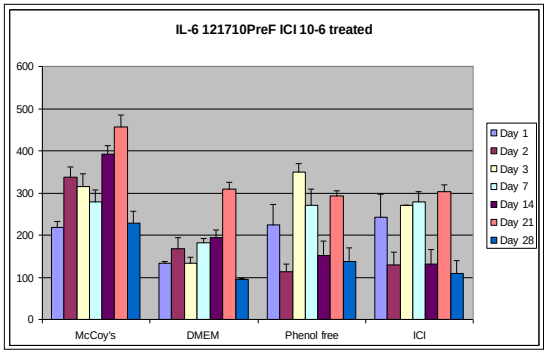
DH2O2
DCCCP
Day 1
Day 2
Day 3
Day 7


IL-6 121710PreF ICI 10-6 treated
600
500
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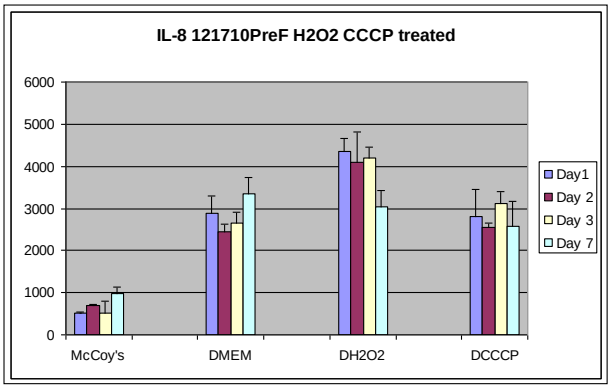
0
McCoy's
DMEM
Phenol free
ICI
Day 1
Day 2
Day 3
Day 7
Day 14
Day 21
Day 28


IL-8 121710PreF H2O2 CCCP treated
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5000
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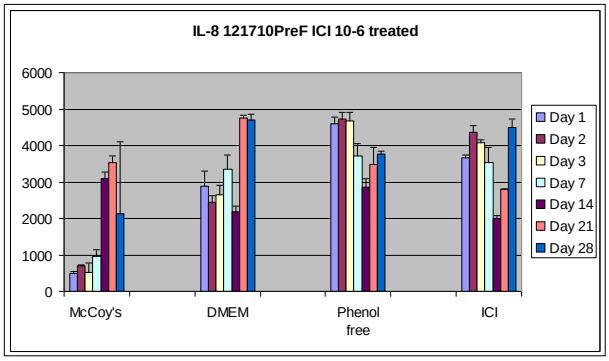
2000
1000
0
McCoy's
DMEM
DH2O2
DCCCP
Day1
Day 2
Day 3
Day 7


IL-8 121710PreF ICI 10-6 treated
6000
5000
4000
3000
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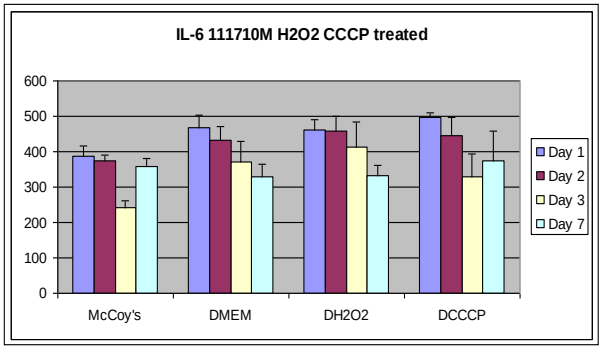
McCoy's
DMEM
Phenol free
ICI
Day 1
Day 2
Day 3
Day 7
Day 14
Day 21
Day 28

D.


IL-6 111710M H2O2 CCCP treated
600
500
400
300
200
100
0
McCoy's
DMEM
DH2O2
DCCCP
Day 1
Day 2
Day 3
Day 7

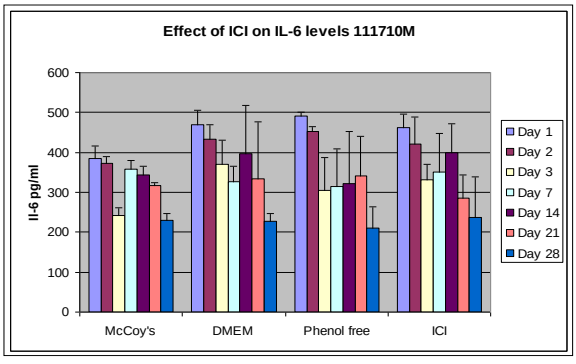

Effect of ICI on IL-6 levels 111710M
Il-6 pg/ml
600
500
400
300
200
100
0
McCoy's
DMEM
Phenol free
ICI
Day 1
Day 2
Day 3
Day 7
Day 14
Day 21
Day 28

Figure 10. Export of IL-6 and IL-8 in human bone marrow stroma in response to stimulation by H_2O_2, CCCP and estrogen deprivation. Measurements of IL-6 and IL-8 in conditioned media from stroma incubated in 24 well tissue culture plates. McCoy's media used to culture stroma to confluence was replaced with DMEM/10% FCS supplemented with H_2O_2, CCCP 100 μM for one hour or in continuous culture with Phenol Red-free media with or without supplemented 10^{-6} M ICI182780. Conditioned media were collected at the times indicated and stored at -80°C, thawed collectively assayed at the same time for IL-6 and IL-8 using a Human IL-6 Elisa Kit from BD & Human CXCL8/IL-8 kit from R&D Systems respectively. Control wells are ones in which McCoy's media was replaced by DMEM/10% FCS.

We used western blots to determine whether hypoxic and oxidative injury resulted in activation of the TGF beta pathway. Figure 11 demonstrates that both CCCP and H2O2 induced phosphorylation of SMAD-3 as a reflection of TGFβ activation. The effect of H_2O_2 appeared to be more intense. These experiments are ongoing.

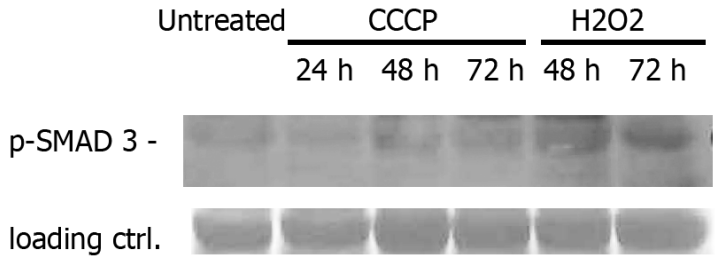


Figure 11. Western blot for phospho-SMAD3 of mouse stromal cells treated with H2O2 and CCCP. Nearly confluent mouse stromal monolayers maintained in 10cm plastic dishes were treated with CCCP and H2O2 100 μM for one hour, washed with PBS and incubated in DMEM 5% FCS for 24, 48 or 72 hours. Cells were lysed in modified radioimmunoprecipitation assay (RIPA) buffer (Santa Cruz Biotechnology) and analyzed by SDS-PAGE. Membranes were probed with antibodies to mouse IL-6 (Abcam Inc.), Cox-2(Cell Signaling), TGF-β(Cell Signaling), phospho-SMAD-2 (Ser 465/467), total SMAD-2, phospho- SMAD-3 (Ser 423/425) and total SMAD-3 (Cell Signaling). Uniform bands from Coomassie Blue-stained membranes were used to verify equal loading. Shown is a membrane demonstrating increased phospho- SMAD-3 staining two and three days after treatments with hypoxic and oxidative stimuli. Other western blots with the other listed antibodies and controls are ongoing.

Once control studies with hypoxic and oxidative injury collected sufficient data to identify doses and times after initial incubation that resulted in quantifiable injury and evidence of secretory senescence in mouse stromal cells, we proceeded to determine if estrogen deprivation induced secretory senescence in stroma. We assessed IL-6 and IL-8 secretion and TGFβ signaling as indicators of secretory senescence. Nearly confluent stromal monolayers were incubated without and with ICI182780, and estrogen receptor alpha (ERα) inhibitor at variable concentrations of 10^{-8}, 10^{-7} and 10^{-6} M for 1, 2, 3, 4, 7, 13, 21 and 28 days in phenol red-free medium. We used 100 μM H_2O_2 incubation and IL-6 secretion after 48 hours as a positive control. Figure 12 demonstrates that deprivation of estrogen induces export of IL- 6 by mouse stroma into the medium after 14 hours. The level of IL-6 peaks after 72 hours, then abates. However, levels remain measurable and significantly higher than those in untreated stroma for the entire four weeks.

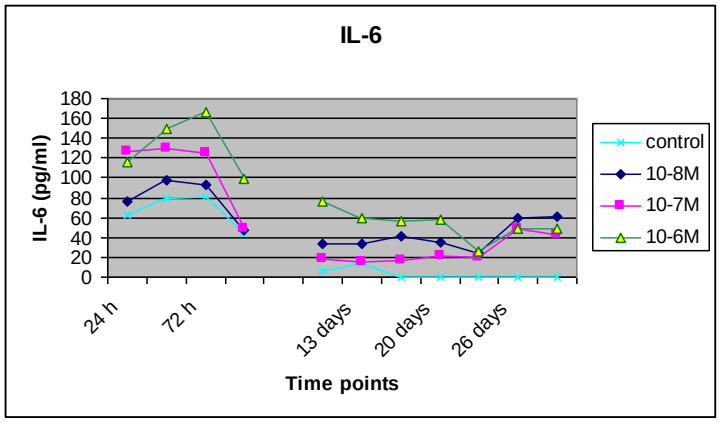


Figure 12. Time and dose effects of ICI182780 on IL-6 in the medium of murine stromal cells. Near confluent stromal monolayers were cultured in 24-well plates in phenol red-free DMEM 10% fetal calf serum with ICI182780 at variable concentrations of 10^{-8}, 10^{-7} and 10^{-6} M for variable times until 4 weeks. Medium and ICI were replenished twice a week. Supernatant samples were collected at 1, 2, 3, 4, 7, 13, 21 and 28 days. The IL-6 levels in the supernatant were determined using an IL-6 ELISA kit (BD Biosciences), according to the manufacturer's instructions. *Columns:* means; *bars:* SE. Experiments were done in triplicate at three samples per point. Data are from one representative experiment.

To determine the effect of estrogen deprivation on activation of the TGFβ pathway and inflammation, we carried out western blots to determine the phosphorylation of SMAD-2 and on TNFα phosphorylation in lysates from stroma deprived of estrogen stimulations. Figure 13 demonstrates that murine stroma incubated with variable concentrations of ICI182780 for 24 and 48 hours in the absence of phenol red induced phosphorylation of phospho-SMAD2, indicative of TGFβ signal pathway activation. Figure 14 demonstrates that estrogen deprivation in murine stroma also induces inflammation as demonstrated by phosphorylation of TNFα.

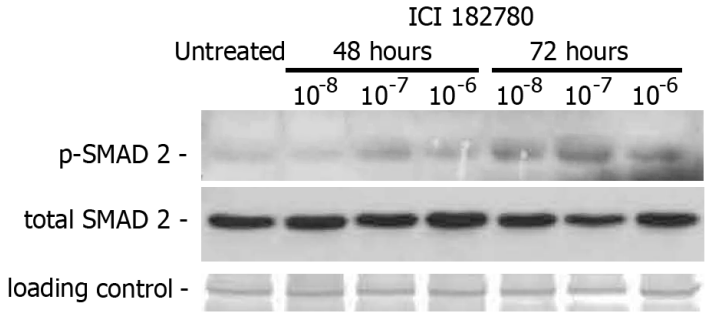


Figure 13. Western blot for phospho-SMAD2 of mouse stromal cells treated with estrogen deprivation. Nearly confluent mouse stromal monolayers maintained in 10 cm plastic dishes were treated with ICI 182780 of 10^{-8}, 10^{-7} and 10^{-6} M for 48 hr and 72 hr were lysed in modified RIPA buffer and analyzed by SDS-PAGE with antibody to mouse phospho-SMAD-2 (Ser 465/467) and total SMAD-2 (Cell Signaling). Uniform bands from Coomassie Blue-stained membranes were used to verify equal loading. The data demonstrate increased phospho- SMAD2 staining with two and three days of estrogen deprivation. Other western blots with antibodies and controls querying TGF beta signaling and COX 2 activation are ongoing.

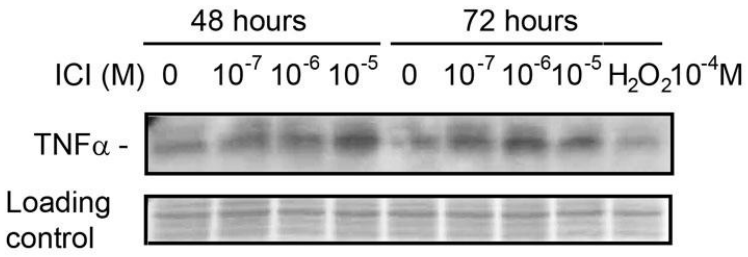


Figure 14. Western blot for phospho-TNFα of mouse stromal cells treated with estrogen deprivation. Nearly confluent mouse stromal monolayers maintained in 10 cm plastic dishes were treated with ICI 182780 of 10^{-7}, 10^{-6} and 10^{-5} M for 48 hr and 72 hr were lysed in modified RIPA buffer and analyzed by SDS-PAGE with antibody to mouse phospho-phospho-TNFα (Cell Signaling). Uniform bands from Coomassie Blue-stained membranes were used to verify equal loading. The data demonstrate increased phospho-TNFα staining with two and three days of estrogen deprivation.

We carried out several controls to demonstrate that ICI182780 at the doses used was effective. Kinetic experiments with MCF-7 estrogen receptor positive and MDA-MB-231 estrogen receptor negative breast cancer cell lines demonstrated that ICI182780 at 10^{-8} M was sufficient to inhibit the proliferation of MCF-7 cells, while, as a negative control, concentrations as high as 10^{-6} M had no effect on the proliferative rate of MDA-MB-231 cells (Figure 15). Western blots of lysates prepared from MCF-7 cells incubated with variable concentrations of ICI182780 for 48 and 72 hours demonstrated the reactive upregulation of ERα in these cells in response to estrogen blockade (Figure 16).

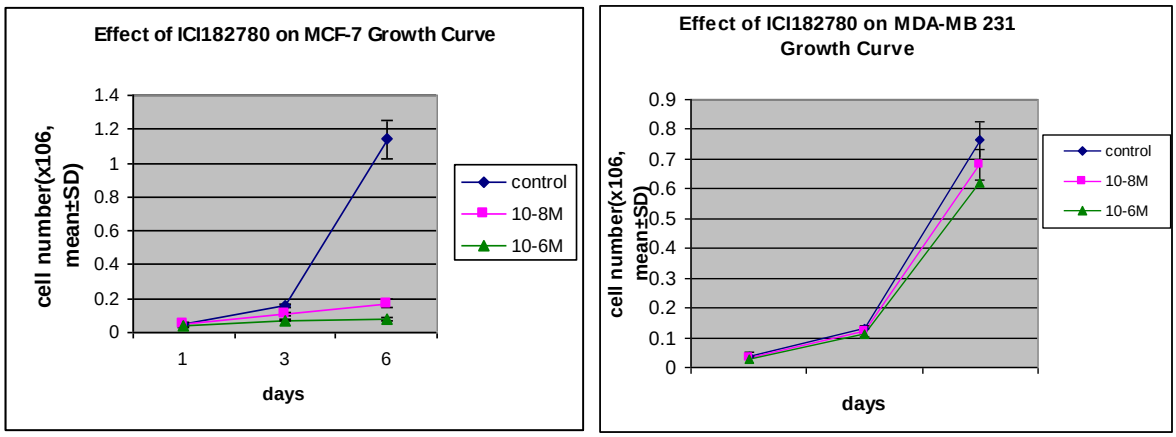


Figure 15. Effect of ICI182780 on MCF-7 and MDA-MB231 cell growth. A total of $5x10^4$ breast cancer cells were cultured in 24 well plates in triplicate wells for 1, 2 and 6 days with ICI182780 was added at the time of initial incubation. Cell number in each well was counted using cell counter. *Bars:* SD.

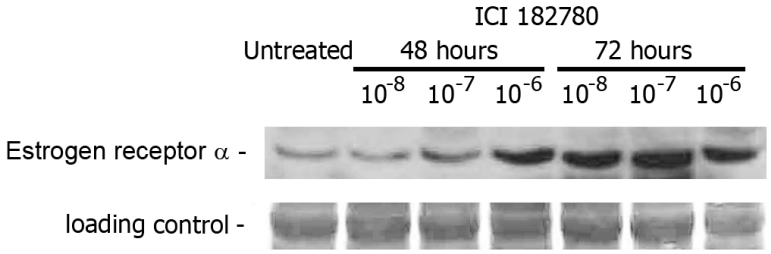


Figure 16. Western blot for ERα in MCF-7 cells treated with estrogen inhibitor ICI182780. Semiconfluent MCF-7 cells maintained in 10 cm plastic dishes were treated with a series of concentrations of ICI 182780 of 10^{-8}, 10^{-7} and 10^{-6} M for 48 hr and 72 hr. Cells were then lysed in modified radioimmunoprecipitation assay (RIPA) buffer (Santa Cruz Biotechnology) and analyzed by SDS-PAGE. Membranes were probed with antibody human ERα (Santa Cruz Biotechnology). Coomassie Blue-stained membranes were used to verify equal loading.

Our goal is to determine if estrogen deprivation-induced stromal secretory senescence deprives bone marrow stroma of its capacity to support dormant colonies in co-culture. The dormancy model we will use involves three components: estrogen sensitive breast cancer cells, partial redifferentiation with FGF-2 that results in re-expression of integrins lost with malignant transformation and fibronectin, a component of bone marrow stroma that initiates specific survival signaling through PI3K when it ligates newly re-expressed integrin α5β1 on breast cancer cells. Figure 17 demonstrates our standard clonogenic assays for dormant colony formation of MCF-7 cells incubated on fibronectin-coated tissue culture plates with FGF-2. The clonogenic potential to form >30 cell growing clones is disrupted by incubation with FGF-2 and the formation of 2-12 cell dormant clones consisting of enlarged, flattened cells with large cytoplasm to nucleus ratios with cortically rearranged f-actin and inactivated RhoA is enabled (Korah, et al., 2004; Barrios and Wieder, 2009). We repeated the assay in preparation for co-incubating these cells with human stromal monolayers before and after estrogen-deprivation-induced secretory senescence.

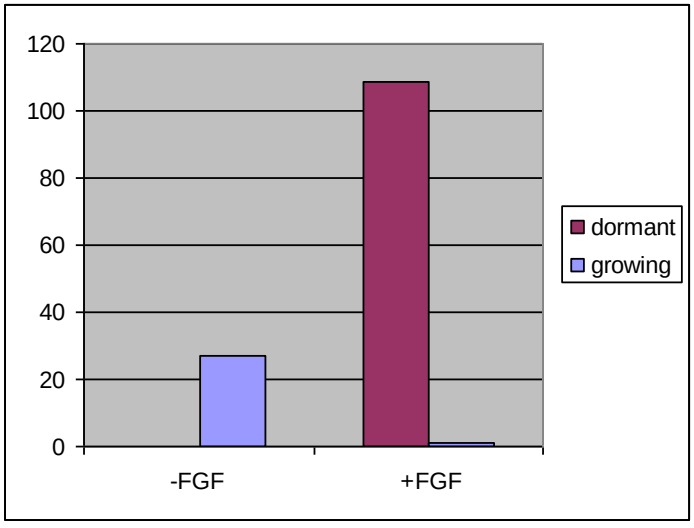


Figure 17. Formation of dormant clones by MCF-7 on Fibronectin with FGF-2. A total of 1,000 MCF-7 cells were incubated on Fibronectin-coated 24-well plates in quadruplicate with and without 10 ng/ml FGF-2 that was added the following day. Six days after FGF-2 addition, growing colonies of >30 cells and dormant clones of 2-12 cells with the distinct appearance of large, spread cells with large cytoplasmic to nucleus ratios were counted. The data demonstrate almost complete disappearance of growing clones and the appearance of dormant clones in the FGF-2 treated wells.

One of the hypotheses we wanted to test was that dormant clones that re-enter the cell cycle promote a self generating progression due to their influence on secretion of inflammatory cytokines in the bone marrow stroma. We proceeded to determine if co-incubation of breast cancer cells with stroma induces secretion of IL-6 and IL-8 into the media. Figure 18 demonstrates that while incubation with optimal concentrations of H_2O_2 induces a statistically significant production of IL-6 in stroma conditioned media, incubation with as few as 30 MCF-7 or MDA-MB-231 cells recapitulates this effect. The response is markedly enhanced by 1,000 cells and reaches a maximum at 3,000 cells co-incubated with stromal monolayers on confluent stromal covered wells on 24 well plates. Figure 19 Demonstrates that incubation of 1,000 or more breast cancer cells with stroma also induce secretion of IL-8 into the conditioned media, albeit not at brisquely as IL-6.

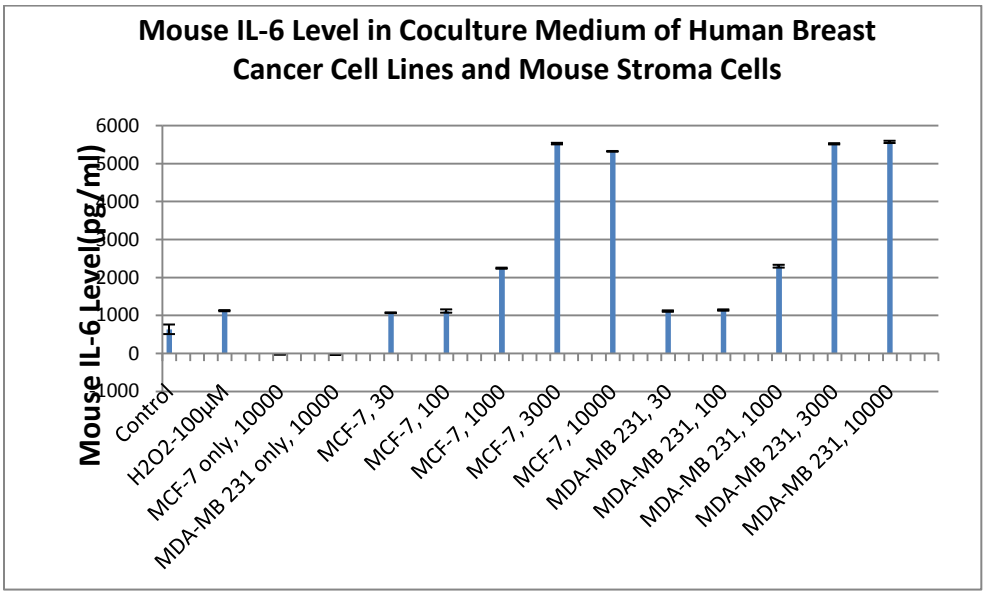


Figure 18. Confluent stromal monolayers were prepared from murine bone marrow as before. At confluence, stroma were treated with H_2O_2 100 μM for an hour, washed once with PBS and media changed to DMEM/5%FCS. Simultaneously, supernatant in triplicate wells was changed to DMEM/5%FCS with variable numbers of MCF-7 and MDA-MB-231 cells and incubated for 48 hours. Supernatant samples were collected 48h and IL-6 levels in the supernatant were determined using an IL-6 ELISA kit (BD Biosciences), according to the manufacturer's instructions. *Columns:* means; *bars:* SE. Experiments were done in triplicate at three samples per point. Data are from one representative experiment.

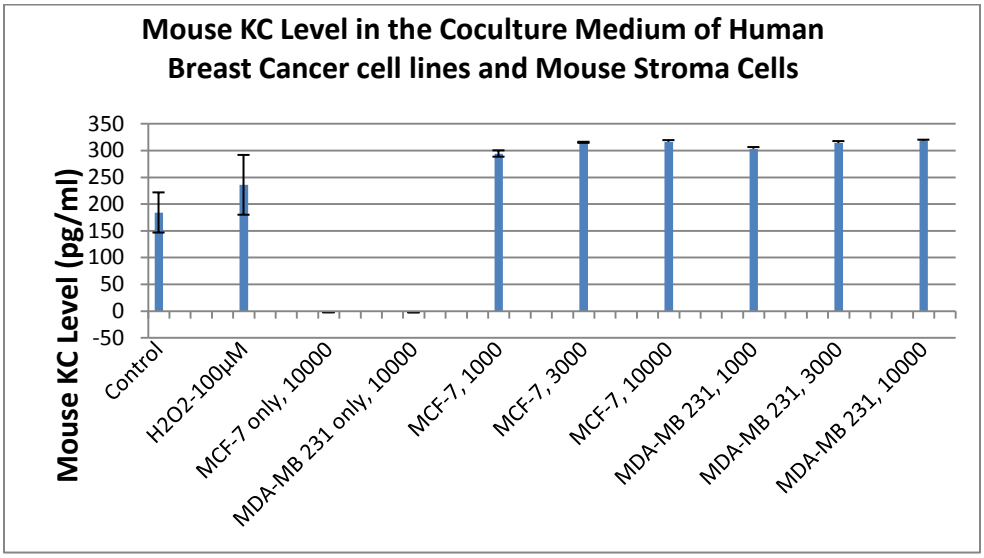


Figure 19. Confluent stromal monolayers were prepared from murine bone marrow as before. At confluence, stroma were treated with H_2O_2 100 μM for an hour, washed once with PBS and media changed to DMEM/5%FCS. Simultaneously, supernatant in triplicate wells was changed to DMEM/5%FCS with variable numbers of MCF-7 and MDA-MB-231 cells and incubated for 48 hours. Supernatant samples were collected 48h and IL-8 levels in the supernatant were determined using an IL-8 (KC) ELISA kit (R&D Systems), according to the manufacturer's instructions. *Columns:* means; *bars:* SE. Experiments were done in triplicate at three samples per point. Data are from one representative experiment.

We investigated the effects of stromal injury on mechanisms of dormant breast cancer reawakening by testing the hypothesis that injured stroma induce osteoclast activation. To determine this effect, we adapted an osteoclast assay to quantitatively measure response of osteoclasts to stimuli. We used BD BioCoat™ Osteologic™ Discs that incorporate a resorbable artificial bone analog in the form of sub-micron calcium phosphate films on transparent quartz substrates. This system can be used as a method for direct assessment of osteoclast activity *in vitro*. We used primary osteoclasts from athymic mouse bone marrows without further purification. Bone marrow was flushed from mouse femurs with 1 ml Osteoclast medium consisting of αMEM containing 15% FBS/Penn/Strep. The cell suspension was diluted to different dilutions with αMEM containing 15% FBS/Penn/Strep, 0.28 mM L-Ascorbic Acid 2-Phosphate and 10 mM β-Glycerophosphate and 1 ml was added on to each Disc placed in a 24 well dish and incubated at 37^o, 5% CO_2, 100% humidity. The medium was changed after 24 hours and then 3 times a week. The osteoclast cultures were maintained for 8-10 days, after which the medium was removed and the discs washed with Milli-Q water. 1ml of bleach solution (~6% NaOCl, ~5.2% NaCl) was added to each well. The solution was pipetted up and down to dislodge the cells. The bleach was aspirated after 5 minutes at room temperature and the discs were washed 3 times with ~2ml of distilled water. The discs were allowed to air dry and examined under the microscope. Figure 20 demonstrates the osteoclast discs and the osteoclast-induced lacunae after an 8-10 day incubation. We demarkated maximal longitudinal diameters in preparation of quantitative assessment of conditioned-media effect on osteoclast activity. Experiments where we incubate bone marrow with conditioned media on the discs for 24 hours prior to the first change of osteoclast media have been ongoing and continue.

MC3T3E1 MC3T3E1 - MCF-7 co-culture Mouse Osteoclasts on Disc

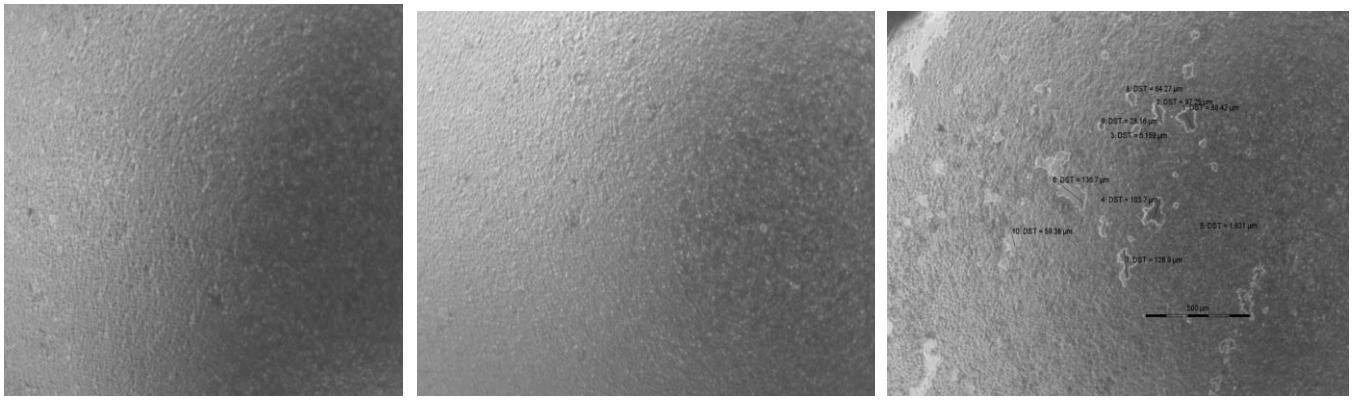


Figure 20 BD BioCoat™ Osteologic™ Discs incubated with Rat Osteoblast cell line MC3T3E1, MC3T3E1 and MCF-7 cells and mouse bone marrow containing osteoclasts. Cells were cultured on discs for 10 days in αMEM containing 15% FBS/Penn/Strep, 0.28 mM L-Ascorbic Acid 2-Phosphate and 10 mM β-Glycerophosphate. Mouse osteoclasts produced numerous, measurable lacunae at a high efficiency while MC3T3 E1 (Preosteoblasts) and MCF-7 did not produce significant numbers of sizes of lacunae. The osteoclast-induced lacunae have longitudinal measurement bars demonstrating that we will be able to quantitate size as well as number differences when we stimulate osteoclasts with stromal conditioned medium.

Another aspect of the project that is highly relevant to our question is the nature of the microenvironment in the bone marrow that supports dormancy of breast cancer cells with the capacity to regrow into recurrent tumors. The nature of this niche has been demonstrated in hematopoietic stem cells to be directly dependent on preosteoblasts lining the bone marrow cavity in the bone marrow stroma. These osteoblasts are necessary for hematopoietic stem cell survival and a genetically induced increase in their number increases the number of hematopoietic stem cells. We hypothesized that osteoblasts also provide a niche for breast cancer repopulating micrometastases in the bone marrow. We proceeded to investigate this possibility in vitro using the rat calvarium preosteoblast line MC3T3E1. We formed monolayers of MC3T3E1 cells as well as monolayers of mouse stromal cells to determine the relative efficiency of MCF-7 human breast cancer cell dormant colony formation. Figure 21 demonstrated the appearance of the osteoblast and stromal monolayers and the presence of growing and dormant MCF-7 breast cancer clones on them after 6 days in coculture. Breast cancer cells were labeled with Invitrogen Cell Tracker fluorescent probes for visualization.

Figure 21. MCF-7 clone formation on various substrata

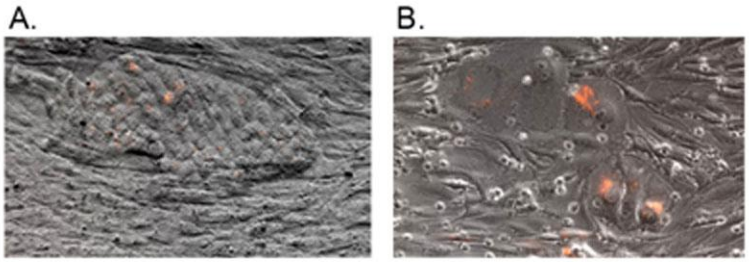


Figure 21. MCF-7 breast cancer cells labeled with Cell Tracker (Invitrogen) were incubated for 6 days on confluent monolayers of rat calvarium preosteoblast line MC3T3E1 (A) and mouse bone marrow stroma (B). **A.** Growing (left) and dormant (right) clones on a monolayer of osteoblasts. **B.** Dormant clones on a monolayer of stroma. All MCF-7 cells were labeled with Cell Tracker cell-labeling solution prior to co-incubation on the various substrata.

We were able to quantitate the number of growing (>30 cells estimated) and dormant (<12 cell estimated) clones after 6 days in culture in a preliminary experiments. While stroma produce their own FGF-2, as we have previously demonstrated, we added exogenous FGF-2 10 ng/ml to half the cultures because osteoblasts were negative for FGF-2 by Western blot. Triplicate wells were counted for numbers of growing and dormant clones. Counting was conducted by switching between phase contrast and fluorescent imaging of the same fields to confirm the morphology of the colony containing fluorescent cells. Growing clones had much lower intensity fluorescence due to dilution of the labels with each cell division, hence accounting for only a fraction of cells with detectable fluorescence. Figure 22 demonstrates the numbers of dormant to growing clones on osteoblasts, stroma and in 10:1 mixture of stroma to osteoblasts.

The ratios of dormant to growing MCF-7 clones are depicted in tables 2-4. Ratios of dormant to growing MCF-7 clones were unchanged ($p>0.05$) regardless of treatment with exogenous FGF-2 on stroma and a mixture of 10:1 stroma:osteoblast monolayers. However, on a monolayer of osteoblasts alone, the ratio of dormant to growing clones was significantly reduced when left untreated with exogenous FGF-2 ($p<0.05$). Error bars indicate ± SD.

Figure 22. The effect of bone marrow stroma and osteoblasts on dormant clone formation

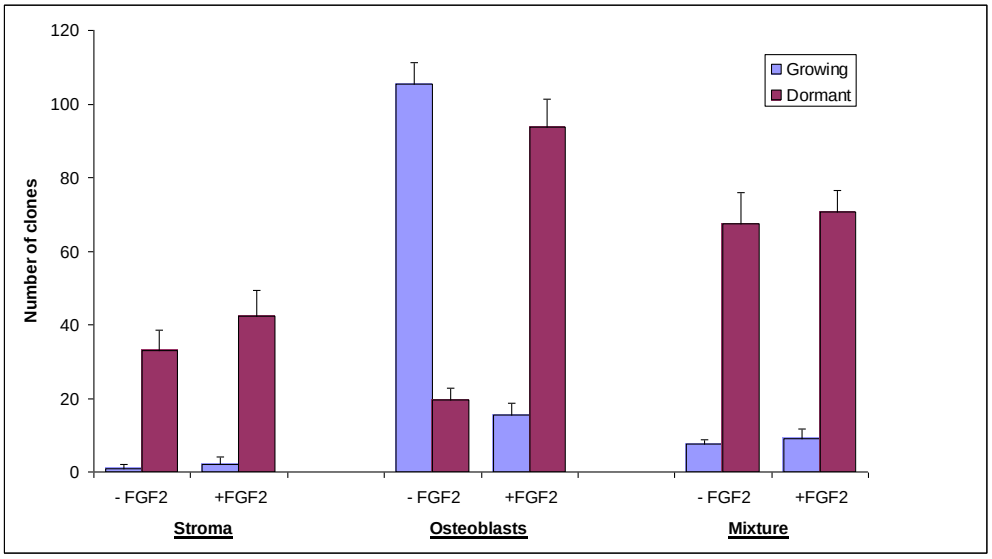


Figure 22. Numbers of growing (>30 cells estimated) and dormant (<12 cell estimated) clones after 6 days in culture of MCF-7 cells incubated on confluent monolayers of athymic mouse bone marrow stroma, on rat calvarium preosteoblast line MC3T3E1 and on a 10:1 mixture of stroma to osteoblast cells. MCF-7 cells were labeled with Cell Tracker (Invitrogen) and colonies were visualized by imaging by both phase contrast and fluorescence microscopy. As previously documented by our group, there were very few growing clones on stroma, with a significant number of dormant clones. The number of dormant clones did not increase with FGF-2 treatment, as expected. Osteoblasts do not produce FGF-2 and consequently, very few dormant clones were observed in its absence. They did support highly efficient growing clone formation. Addition of FGF-2 to the osteoclast cocultures induced a robust dormant clone formation at double the efficiency of that on stroma. Addition of 1/10 osteoblasts to a mixed stromal monolayer also increased the efficiency of dormant clone formation by nearly a factor of 2 while stimulating the formation of growing clones as well.

Table 2. Number of growing MCF-7 clones on various substrata without exogenous FGF-2

	Number of growing clones	Change compared to plastic and fibronectin	P-value compared to plastic	P-value compared to fibronectin
Plastic	66		-	0.018
Fibronectin	39		0.018	-
Osteoblasts	105	increased	0.001	<0.001
Stroma	1	decreased	<0.001	0.004
Mixture	8	decreased	<0.001	0.008

Without exogenous FGF-2, osteoblasts promote growth of MCF-7 cells. Stromal and mixed cultures inhibit growth.

Table 3. Number of dormant MCF-7 clones on osteoblasts and stroma

	Number of dormant clones without FGF-2	Number of dormant clones with FGF-2	P-value, without versus with FGF-2
Osteoblasts	20	94	<0.001
Stroma	33	42	0.15

Osteoblasts require exogenous FGF-2 to induce dormancy of MCF-7 cells. Bone marrow stroma does not require it, likely due to endogenous FGF-2 being produced and released by stromal cells.

Table 4. Number of dormant MCF-7 clones on osteoblasts, stroma, and mixture

	Number of dormant clones without FGF-2	Number of dormant clones with FGF-2
Stroma	33 *	42 * *
Osteoblasts	20 *	94 *
Mixture	67	71

* Indicates statistical significance ($p<0.05$)

Without exogenous FGF-2, osteoblasts support fewer dormant clones than stroma. However, when exogenous FGF-2 is provided, the presence of osteoblasts, either alone or in the 10:1 stroma to osteoblast mixture, significantly increases the number of dormant clones.

These data suggest that osteoblasts alone do not appear to support dormancy of breast cancer cells when FGF-2 is absent. However, when provided with FGF2 either exogenously with recombinant FGF2 or through mixing with bone marrow stromal cells, osteoblasts appear to promote increased dormant clone formation.

Since osteoblasts in the bone marrow niche are in the presence of FGF-2 produced by contiguous stromal cells, the scenario observed in the mixing experiments is likely more representative than that of osteoblast co-cultures alone. These mixing experiments suggests that the presence of osteoblasts in a stromal microenvironment increases the rate of dormancy compared with that induced by stroma alone. Similarly, osteoblasts increase dormant colony formation in pure cocultures with exogenous FGF-2 added. The mechanism of osteoblast induced greater number of dormant clone formation as well as that of increased growing clone formation will be important to determine. We have demonstrated that increased survival of dormant clones on bone marrow stroma is in part due to activation of the PI3 kinase pathway. It is likely that activation of a parallel pathway is induced by adhesion molecule signaling activated by osteoblast ligation, but these data are yet to be determined.

It will also be important to determine the capacity of dormant clones in these co-cultures to repopulate a tumor and whether the enhanced dormant clone formation on osteoblasts correlates with greater repopulating capacity of those cells.

KEY RESEARCH ACCOMPLISHMENTS:

- Developed *in vitro* stromal injury model in mouse and human bone marrow stroma
- Confirmed that H_2O_2-induced oxidative damage and CCCP-induced hypoxic damage can induce IL-6 and variably IL-8 export by human stroma
- Demonstrated that estrogen deprivation of human bone marrow stroma induces export of IL-6 and IL-8 in female, premenopausal stroma, but possibly not in male stroma
- Adapted a co-culture model of breast cancer cells with injured stroma to determine the effect on stromal capacity to support dormancy
- Demonstrated that breast cancer cells co-cultivated with stroma induce IL-6 and IL-8 secretion in a dose-dependent manner and possibly contribute to a positive feedback loop to promote recurrent tumor growth once cycle activated
- Adapted an osteoclast activation model to determine the effect of injured stroma to activate osteoclasts
- Adapted a preosteoblast co-culture model to determine the role of osteoblasts in the micrometastatic cell niche responsible for recurrence of the dormant breast cancer

REPORTABLE OUTCOMES:

Robert Wieder, Tanya Dasgupta, Haiyan Lu, Cassandre Noel. Estrogen deprivation induces secretory senescence in human bone marrow stromal cells that support breast cancer dormancy. The Department of Defense Breast Cancer Research Program Meeting, "Era of Hope". Orlando, FL, August 2011, #8-48

C**ONCLUSIONS:**

The data demonstrate, for the first time, that bone marrow stroma develop characteristics described in the state called secretory senescence. This cellular condition has been described in other systems and consist of an injured state that, among other traits, is associated with inflammatory signaling, activation of the TGFβ and inflammatory pathways and secretion of inflammatory cytokines. We hypothesized that estrogen deprivation may be one mechanism that can induce secretory senescence in the bone marrow stroma, and effect that may be universal in menopause. This is potentially of great significance, as the secreted inflammatory cytokines may induce breast cancer micrometastases that have been dormant in the stromal microenvironment for extended periods to begin proliferating again and result in incurable recurrent disease.

We tested the hypothesis that estrogen deprivation induces secretory senescence in bone marrow stroma and demonstrated that these stromal cells begin to secrete IL-6 and activate TGFβ and inflammatory signaling in response to estrogen deprivation. We also demonstrated that breast cancer cells induce the secretion of these inflammatory cytokines when incubated with stroma in a dose dependent manner, supporting the hypothesis that once cycle activated, micrometastases provide a positive feedback through stromal cytokine secretion in progression to growing disease. Anti-inflammatory treatment of senescent stroma with aspirin and indomethacin are experiments ongoing in our lab as part of this project to determine if they can reverse this effect.

We also demonstrated a capacity of damaged stroma to induce increased osteocalst activity in preliminary observations and those experiments continue. We showed that preosteoblasts, purported members of the hematopoietic stem cell niche, support the efficiency of dormant breast cancer clone formation as well as the efficiency of growing colony formation in *in vitro* co-culture experiments.

We are continuing experiments started in this project and expanding the scope to determine the mechanisms of the effects of estrogen-induced stromal senescence and methods to disrupt it to restore its capacity to support dormancy.

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Korah R, Boots M, and Wieder R. (2004) Integrin $\alpha 5\beta 1$ promotes survival of growth-arrested breast cancer cells: an *in vitro* paradigm for breast cancer dormancy in bone marrow. Cancer Research 64: 4514-4522.

Barrios, J and Wieder R. (2009) Dual FGF-2 and intergrin α5β1 signaling mediate GRAF-induced RhoA inactivation in a model of breast cancer dormancy. Cancer Microenvironment 2:33–47.

Lo Celso C, Fleming HE, Wu JW, et al. Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. *Nature.* 2009;457(7225):92-96.

APPENDICES:

None

SUPPORTING DATA:

All data appear in the body.